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(71) Applicant: **Ortho-Clinical Diagnostics, Inc.**
Rochester, NY 14626-5101 (US)

(72) Inventor: **Jatkoe, Tim**
La Jolla, CA 92037-4335 (US)

(74) Representative: **Mercer, Christopher Paul et al**
Carpmaels & Ransford
43, Bloomsbury Square
London WC1A 2RA (GB)

(54) Method of selecting a portfolio of markers for use in a diagnostic application

(57) Methods of selecting a portfolio of markers for use in a diagnostic applications include defining diagnostic parameters, establishing a relationship among the parameters so that they are optimized, and selecting an optimal group of markers for the diagnostic application. The diagnostic parameters can include a measure of the relative degree of expression of a gene, a measure of the variation in the measurement of the degree of expression of the gene, and the relationship between the diagnostic and discriminating parameters can be a mean variance relationship.

Machines programmed to conduct the method and articles that comprise instructions for their operation are further aspects of the invention.

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Description

BACKGROUND

[0001] This application claims the benefit of U.S Provisional Application No.60/368,790 filed on March 29, 2002.

[0002] The invention relates to the selection of portfolios of diagnostic markers.

[0003] A few single gene diagnostic markers such as her-2-neu are currently in use. Usually, however, diseases are not easily diagnosed with molecular diagnostics for one particular gene. Multiple markers are often required and the number of such markers that may be included in a assay based on differential gene modulation can be large, even in the hundreds of genes. It is desirable to group markers into portfolios so that the most reliable results are obtained using the smallest number of markers necessary to obtain such a result. This is particularly true in assays that contain multiple steps such as nucleic acid amplification steps.

SUMMARY OF THE INVENTION

[0004] The invention is a method of selecting a portfolio of markers for use in a diagnostic application in which diagnostic parameters are defined, a relationship among the parameters is established so that they are optimized, and the relationship is used to select an optimal group of markers for the diagnostic application.

[0005] In another aspect of the invention, the diagnostic parameters include a measure of the relative degree of expression of a gene, a measure of the variation in the measurement of the degree of expression of the gene, and the relationship between the diagnostic and discriminating parameters is a mean variance relationship.

[0006] Machines programmed to conduct the inventive method and articles that comprise instructions for their operation are further aspects of the invention.

DETAILED DESCRIPTION

[0007] The methods of this invention can be used in conjunction with any method for determining the gene expression patterns of relevant cells as well as protein based methods of determining gene expression. Preferred methods for establishing gene expression profiles include determining the amount of RNA that is produced by a gene that can code for a protein or peptide. This is accomplished by reverse transcriptase PCR (RT-PCR), competitive RT-PCR, real time RT-PCR, differential display RT-PCR, Northern Blot analysis and other related tests. While it is possible to conduct these techniques using individual PCR reactions, it is best to amplify copy DNA (cDNA) or copy RNA (cRNA) produced from mRNA and analyze it via microarray. A number of different array configurations and methods for their production are known to those of skill in the art and are described in U.S. Patents such as: 5,445,934; 5,532,128; 5,556,752; 5,242,974; 5,384,261; 5,405,783; 5,412,087; 5,424,186; 5,429,807; 5,436,327; 5,472,672; 5,527,681; 5,529,756; 5,545,531; 5,554,501; 5,561,071; 5,571,639; 5,593,839; 5,599,695; 5,624,711; 5,658,734; and 5,700,637; the disclosures of which are incorporated herein by reference.

[0008] Microarray technology allows for the measurement of the steady-state mRNA level of thousands of genes simultaneously thereby presenting a powerful tool for identifying effects such as the onset, arrest, or modulation of uncontrolled cell proliferation. Two microarray technologies are currently in wide use. The first are cDNA arrays and the second are oligonucleotide arrays. Although differences exist in the construction of these chips, essentially all downstream data analysis and output are the same. The product of these analyses are typically measurements of the intensity of the signal received from a labeled probe used to detect a cDNA sequence from the sample that hybridizes to a nucleic acid sequence at a known location on the microarray. Typically, the intensity of the signal is proportional to the quantity of cDNA, and thus mRNA, expressed in the sample cells. A large number of such techniques are available and useful. Preferred methods for determining gene expression can be found in US Patents 6,271,002 to Linsley, et al.; 6,218,122 to Friend, et al.; 6,218,114 to Peck, et al.; and 6,004,755 to Wang, et al., the disclosure of each of which is incorporated herein by reference.

[0009] Analysis of the expression levels is conducted by comparing such intensities. This is best done by generating a ratio matrix of the expression intensities of genes in a test sample versus those in a control sample. For instance, the gene expression intensities from a diseased tissue can be compared with the expression intensities generated from normal tissue of the same type (e.g., diseased colon tissue sample vs. normal colon tissue sample). A ratio of these expression intensities indicates the fold-change in gene expression between the test and control samples.

[0010] Modulated genes are those that are differentially expressed as up regulated or down regulated in non-normal cells. Up regulation and down regulation are relative terms meaning that a detectable difference (beyond the contribution of noise in the system used to measure it) is found in the amount of expression of the genes relative to some baseline. In this case, the baseline is the measured gene expression of a normal cell. The genes of interest in the non-normal cells are then either up regulated or down regulated relative to the baseline level using the same measurement

method.

[0011] Preferably, levels of up and down regulation are distinguished based on fold changes of the intensity measurements of hybridized microarray probes. For example, in the case in which a 1.5 fold or more difference is used to make such distinctions, the diseased cell is found to yield at least 1.5 times more, or 1.5 times less intensity than the normal cells.

[0012] Other methods of making distinctions are available. For example, statistical tests can be used to find the genes most significantly different between diverse groups of samples. The Student's t-test is an example of a robust statistical test that can be used to find significant differences between two groups. The lower the p-value, the more compelling the evidence that the gene is showing a difference between the different groups. Nevertheless, since microarrays measure more than one gene at a time, tens of thousands of statistical tests may be asked at one time. Because of this, there is likelihood to see small p-values just by chance and adjustments for this using a Sidak correction as well as a randomization/permutation experiment can be made.

[0013] A p-value less than .05 by the t-test is evidence that the gene is significantly different. More compelling evidence is a p-value less then .05 after the Sidak correct is factored in. For a large number of samples in each group, a p-value less than 0.05 after the randomization/ permutation test is the most compelling evidence of a significant difference.

[0014] Genes can be grouped so that information obtained about the set of genes in the group provides a sound basis for making clinically relevant judgments such as a diagnosis, prognosis, or treatment choice. These sets of genes make up the portfolios of the invention. As with most diagnostic markers, it is often desirable to use the fewest number of markers sufficient to make a correct medical judgment. This prevents a delay in treatment pending further analysis as well as inappropriate use of time and resources. Preferred optimal portfolio is one that employs the fewest number of markers for making such judgments while meeting conditions that maximize the probability that such judgments are indeed correct. These conditions will generally include sensitivity and specificity requirements. In the context of microarray based detection methods, the sensitivity of the portfolio can be reflected in the fold differences exhibited by a gene's expression in the diseased or aberrant state relative to the normal state. The detection of the differential expression of a gene is sensitive if it exhibits a large fold change relative to the expression of the gene in another state. Another aspect of sensitivity is the ability to distinguish signal from noise. For example, while the expression of a set of genes may show adequate sensitivity for defining a given disease state, if the signal that is generated by one (e.g., intensity measurements in microarrays) is below a level that easily distinguished from noise in a given setting (e.g., a clinical laboratory) then that gene should be excluded from the optimal portfolio. A procedure for setting conditions such as these that define the optimal portfolio can be incorporated into the inventive methods.

[0015] Specificity can be reflected in statistical measurements of the correlation of the signaling of gene expression with the condition of interest. If the differential expression of a set of genes is observed to produce a large fold change but they do so for a number of conditions other than the condition of interest (e.g. multiple disease states) then the gene expression profile for that set of genes is non-specific. Statistical measurements of correlation of data or the degree of consistency of data such as standard deviation, correlation coefficients, and the like can be a used as such measurements. In considering a group of genes for inclusion in a portfolio, a small standard deviation in expression measurements correlates with greater specificity. Genes that display similar expression patterns may be co-regulated by an identical factor that pushes the genes in the same direction. If this factor is sufficient but not necessary for classifying a sample, then these genes will fail to correctly identify a sample if the markers are all related to this single factor. Diversification then results in selecting as few markers as possible, yet covers as many different optimal expression patterns that are contained in the data set

[0016] In the method of the invention, a group of genetic markers is selected for use in diagnostic applications. These groups of markers are "portfolios". Diagnostic applications include the detection or identification of a disease state or condition of a subject, determining the likelihood that a subject will contract a given disease or condition, determining the likelihood that a subject with a disease or condition will respond to therapy, determining the prognosis of a subject with a disease or condition (or its likely progression or regression), and determining the effect of a treatment on a subject with a disease or condition. For example, the method can be used to establish portfolios for detecting the presence or likelihood of a subject contracting colon cancer or the likelihood that such a subject will respond favorably to cytotoxic drugs.

[0017] The portfolios selected by the method of the invention contain a number and type of markers that assure accurate and precise results and are economized in terms of the number of genes that comprise the portfolio. The method of the invention can be used to establish optimal gene expression portfolios for any disease, condition, or state that is concomitant with the expression of multiple genes. An optimal portfolio in the context of the instant invention refers to a gene expression profile that provides an assessment of the condition of a subject (based upon the condition for which the analysis was undertaken) according to predetermined standards of at least two of the following parameters: accuracy, precision, and number of genes comprising the portfolio.

[0018] Most preferably, the markers employed in the portfolio are nucleic acid sequences that express mRNA

("genes"). Expression of the markers may occur ordinarily in a healthy subject and be more highly expressed or less highly expressed when an event that is the object of the diagnostic application occurs. Alternatively, expression may not occur except when the event that is the object of the diagnostic application occurs.

[0019] Marker attributes, features, indicia, or measurements that can be compared to make diagnostic judgments are diagnostic parameters used in the method. Indicators of gene expression levels are the most preferred diagnostic parameters. Such indicators include intensity measurements read from microarrays, as described above. Other diagnostic parameters are also possible such as indicators of the relative degree of methylation of the markers.

[0020] Distinctions are made among the diagnostic parameters through the use of mathematical/statistical values that are related to each other. The preferred distinctions are mean signal readings indicative of gene expression and measurements of the variance of such readings. The most preferred distinctions are made by use of the mean of signal ratios between different group readings (e.g., microarray intensity measurements) and the standard deviations of the signal ratio measurements. A great number of such mathematical/statistical values can be used in their place such as return at a given percentile.

[0021] A relationship among diagnostic parameter distinctions is used to optimize the selection of markers useful for the diagnostic application. Typically, this is done through the use of linear or quadratic programming algorithms. However, heuristic approaches can also be applied or can be used to supplement input data selection or data output. The most preferred relationship is a mean-variance relationship such as that described in *Mean-Variance Analysis in Portfolio Choice and Capital Markets* by Harry M. Markowitz (Frank J. Fabozzi Associates, New Hope, PA: 2000, ISBN: 1-883249-75-9) which is incorporated herein by reference. The relationship is best understood in the context of the selection of stocks for a financial investment portfolio. This is the context for which the relationship was developed and elucidated.

[0022] The investor looking to optimize a portfolio of stocks can select from a large number of possible stocks, each having a historical rate of return and a risk factor. The mean variance method uses a critical line algorithm of linear programming or quadratic programming to identify all feasible portfolios that minimize risk (as measured by variance or standard deviation) for a given level of expected return and maximize expected return for a given level of risk. When standard deviation is plotted against expected return an efficient frontier is generated. Selection of stocks along the efficient frontier results in a diversified stock portfolio optimized in terms of return and risk.

[0023] When the mean variance relationship is used in the method of the instant invention, diagnostic parameters such as microarray signal intensity and standard deviation replace the return and risk factor values used in the selection of financial portfolios. Most preferably, when the mean variance relationship is applied, a commercial computer software application such as the "Wagner Associates Mean-Variance Optimization Application", referred to as "Wagner Software" throughout this specification. This software uses functions from the "Wagner Associates Mean-Variance Optimization Library" to determine an efficient frontier and optimal portfolios in the Markowitz sense. Since such applications are made for financial applications, it may be necessary to preprocess input data so that it can conform to conventions required by the software. For example, when Wagner Software is employed in conjunction with microarray intensity measurements the following data transformation method is employed.

[0024] A relationship between each genes baseline and experimental value must first be established. The preferred process is conducted as follows. A baseline class is selected. Typically, this will comprise genes from a population that does not have the condition of interest. For example, if one were interested in selecting a portfolio of genes that are diagnostic for breast cancer, samples from patients without breast cancer can be used to make the baseline class. Once the baseline class is selected, the arithmetic mean and standard deviation is calculated for the indicator of gene expression of each gene for baseline class samples. This indicator is typically the fluorescent intensity of a microarray reading. The statistical data computed is then used to calculate a baseline value of (X*Standard Deviation + Mean) for each gene. This is the baseline reading for the gene from which all other samples will be compared. X is a stringency variable selected by the person formulating the portfolio. Higher values of X are more stringent than lower. Preferably, X is in the range of .5 to 3 with 2 to 3 being more preferred and 3 being most preferred.

[0025] Ratios between each experimental sample (those displaying the condition of interest) versus baseline readings are then calculated. The ratios are then transformed to base 10 logarithmic values for ease of data handling by the software. This enables down regulated genes to display negative values necessary for optimization according to the Markman mean-variance algorithm using the Wagner Software.

[0026] The preprocessed data comprising these transformed ratios are used as inputs in place of the asset return values that are normally used in the Wagner Software when it is used for financial analysis purposes.

[0027] Once an efficient frontier is formulated, an optimized portfolio is selected for a given input level (return) or variance that corresponds to a point on the frontier. These inputs or variances are the predetermined standards set by the person formulating the portfolio. Stated differently, one seeking the optimum portfolio determines an acceptable input level (indicative of sensitivity) or a given level of variance (indicative of specificity) and selects the genes that lie along the efficient frontier that correspond to that input level or variance. The Wagner Software can select such genes when an input level or variance is selected. It can also assign a weight to each gene in the portfolio as it would for a

stock in a stock portfolio.

[0028] Determining whether a sample has the condition for which the portfolio is diagnostic can be conducted by comparing the expression of the genes in the portfolio for the patient sample with calculated values of differentially expressed genes used to establish the portfolio. Preferably, a portfolio value is first generated by summing the multiples of the intensity value of each gene in the portfolio by the weight assigned to that gene in the portfolio selection process. A boundary value is then calculated by (Y*standard deviation + mean of the portfolio value for baseline groups) where Y is a stringency value having the same meaning as X described above. A sample having a portfolio value greater than the boundary value of the baseline class is then classified as having the condition. If desired, this process can be conducted iteratively in accordance with well known statistical methods for improving confidence levels.

[0029] Optionally one can reiterate this process until best prediction accuracy is obtained.

[0030] The process of portfolio selection and characterization of an unknown is summarized as follows:

1. Choose baseline class.
2. Calculate mean, and standard deviation of each gene for baseline class samples.
3. Calculate (X*Standard Deviation + Mean) for each gene. This is the baseline reading from which all other samples will be compared. X is a stringency variable with higher values of X being more stringent than lower.
4. Calculate ratio between each Experimental sample versus baseline reading calculated in step 3.
5. Transform ratios such that ratios less than 1 are negative (eg.using Log base 10). (Down regulated genes now correctly have negative values necessary for MV optimization).
6. These transformed ratios are used as inputs in place of the asset returns that are normally used in the software application.
7. The software will plot the efficient frontier and return an optimized portfolio at any point along the efficient frontier.
8. Choose a desired return or variance on the efficient frontier.
9. Calculate the Portfolio's Value for each sample by summing the multiples of each gene's intensity value by the weight generated by the portfolio selection algorithm.
10. Calculate a boundary value by adding the mean Portfolio Value for Baseline groups to the multiple of Y and the Standard Deviation of the Baseline's Portfolio Values. Values greater than this boundary value shall be classified as the Experimental Class.
11. Optionally one can reiterate this process until best prediction accuracy is obtained.

[0031] A second portfolio can optionally be created by reversing the baseline and experimental calculation. This creates a new portfolio of genes which are up-regulated in the original baseline class. This second portfolio's value can be subtracted from the first to create a new classification value based on multiple portfolios.

[0032] Another useful method of pre-selecting genes from gene expression data so that it can be used as input for a process for selecting a portfolio is based on a threshold given by

$$1 \leq \left| \frac{(\mu_t - \mu_n)}{(\sigma_t + \sigma_n)} \right|,$$

where μ_t is the mean of the subset known to possess the disease or condition, μ_n is the mean of the subset of normal samples, and $\sigma_t + \sigma_n$ represent the combined standard deviations. A signal to noise cutoff can also be used by pre-selecting the data according to a relationship such as

$$0.5 \leq \left| \frac{(\mu_t - MAX_n)}{(\sigma_t + \sigma_n)} \right|.$$

This ensures that genes that are pre-selected based on their differential modulation are differentiated in a clinically significant way. That is, above the noise level of instrumentation appropriate to the task of measuring the diagnostic parameters. For each marker pre-selected according to these criteria, a matrix is established in which columns represents samples, rows represent markers and each element is a normalized intensity measurement for the expression of that marker according to the relationship:

$$\left| \frac{(\mu_t - I)}{\mu_t} \right|$$

where I is the intensity measurement.

[0033] Using this process of creating input for financial portfolio software make also allows one to set additional boundary conditions to define the optimal portfolios. For example, portfolio size can be limited to a fixed range or number of markers. This can be done either by making data pre-selection criteria more stringent (e.g,

$$.8 \leq \left| \frac{(\mu_t - MAX_n)}{(\sigma_t + \sigma_n)} \right|$$

instead of

$$\left| 0.5 \leq \frac{(\mu_t - MAX_n)}{(\sigma_t + \sigma_n)} \right|)$$

or by using programming features such as restricting portfolio size. One could, for example, set the boundary condition that the efficient frontier is to be selected from among only the optimal 10 genes. One could also use all of the genes pre-selected for determining the efficient frontier and then limit the number of genes selected (e.g., no more than 10).

[0034] The process of selecting a portfolio can also include the application of heuristic rules. Preferably, such rules are formulated based on biology and an understanding of the technology used to produce clinical results. More preferably, they are applied to output from the optimization method. For example, the mean variance method of portfolio selection can be applied to microarray data for a number of genes differentially expressed in subjects with breast cancer. Output from the method would be an optimized set of genes that could include some genes that are expressed in peripheral blood as well as in diseased breast tissue. If sample used in the testing method are obtained from peripheral blood and certain genes differentially expressed in instances of breast cancer could also be differentially expressed in peripheral blood, then a heuristic rule can be applied in which a portfolio is selected from the efficient frontier excluding those that are differentially expressed in peripheral blood. Of course, the rule can be applied prior to the formation of the efficient frontier by, for example, applying the rule during data pre-selection.

[0035] Other heuristic rules can be applied that are not necessarily related to the biology in question. For example, one can apply the rule that only a given percentage of the portfolio can be represented by a particular gene or genes. Commercially available software such as the Wagner Software readily accommodates these types of heuristics. This can be useful, for example, when factors other than accuracy and precision (e.g., anticipated licensing fees) have an impact on the desirability of including one or more genes.

[0036] Other relationships aside from the mean-variance relationship can be used in the method of the invention provided that they optimize the portfolio according to predetermined attributes such as assay accuracy and precision. Two examples are the Martin simultaneous equation approach (Elton, Edwin J. and Martin J. Gruber (1987), *Modern Portfolio Theory Investment Analysis,* Third Edition, John Wiley, New York, 1987) and Genetic Algorithms (Davis, L., (1989), *Adapting Operator Probabilities in Genetic Algorithms,* in *Proceedings of the Third International Conference on Genetic Algorithms*, Morgan Kaufmann: San Mateo, pp. 61-69). There are also many ways to adapt the mean-variance relationship to handle skewed data such as where a marker detection technology exhibits a known bias. These include, for example, the Semi-Deviation method in which the square root of the average squared (negative) deviation from a reference signal and includes only those signal values that fall below the reference signal.

[0037] Articles of this invention include representations of the gene expression profiles that make up the portfolios useful for treating, diagnosing, prognosticating, and otherwise assessing diseases. These representations are reduced to a medium that can be automatically read by a machine such as computer readable media (magnetic, optical, and the like). The articles can also include instructions for assessing the gene expression profiles in such media. For example, the articles may comprise a CD ROM having computer instructions for comparing gene expression profiles of the portfolios of genes described above. The articles may also have gene expression profiles digitally recorded therein so that they may be compared with gene expression data from patient samples. Alternatively, the profiles can be recorded in different representational format. A graphical recordation is one such format.

[0038] Different types of articles of manufacture according to the invention are media or formatted assays used to reveal gene expression profiles. These can comprise, for example, microarrays in which sequence complements or probes are affixed to a matrix to which the sequences indicative of the genes of interest combine creating a readable determinant of their presence. When such a microarray contains an optimized portfolio great savings in time, process steps, and resources are attained by minimizing the number of cDNA or oligonucleotides that must be applied to the substrate, reacted with the sample, read by an analyzer, processed for results, and (sometimes) verified.

[0039] Other articles according to the invention can be fashioned into reagent kits for conducting hybridization, amplification, and signal generation indicative of the level of expression of the genes in the portfolios established through

the method of the invention. Kits made according to the invention include formatted assays for determining the gene expression profiles. These can include all or some of the materials needed to conduct the assays such as reagents and instructions.

EXAMPLES

Example 1: Producing an Optimized Portfolio

[0040] Gene expression data was recently produced from tissue samples representative of eleven different types of cancers. The data was published in *Cancer Research* 61: 7388-7393, 2001 and http://carrier.gnf.org/welsh/epican/. *See,* Andrew I. Su et al., "Molecular Classification of Human Carcinomas by Use of Gene Expression Signatures."The data included intensity measurements obtained with the use of an "U95"a oligonucleotide microarray (commercially available from Affymetrix, Inc.).

[0041] Measurements of the expression of genes from the published data (fluorescent intensity measurements) was used to select optimum gene expression portfolios for a panel of markers to determine whether a circulating cell is indicative of the presence of breast cancer, prostate cancer, ovarian cancer, colorectal cancer, or lung cancer. Such circulating cells would preferably be epithelial cells.

[0042] The data in the study was collected from the following samples: 24 adenocarcinomas, 12 infilitrating ductal breast adenocarcenomas, 21 colorectal adenocarcinomas, 23 ovarian adenocarcinomas, 25 lung carcinomas, and data from the following additional samples: 19 prostate adenocarcinomas, 12 breast carinomas, 13 colon carcinomas, 13 ovarian carcinomas, 13 ovarian carcinomas, and 89 lung carcenomas.

[0043] Using intensity readings from a collection of normal samples as the baseline class, the arithmetic mean, and standard deviation of each gene were calculated followed by a calculation of the value (X*Standard Deviation + Mean) for each gene. The stringency variable, X, was assigned a value of 3 in this case. Ratios were then calculated between each Experimental sample described in the study versus the baseline value calculations. The ratios were transformed into common logarithms. These values were then used as the input values for the Wagner Software.

[0044] This procedure selected an efficient frontier along which a minimum set of markers for each tumor type that have the lowest amount of variation for a selected level of differential (chosen at the best signal to noise ratio point). Optimization by the software resulted in the selection of a portfolio of 24 genes including 2 for prostate cancer, 5 for breast cancer, 6 for colon cancer, 2 for ovarian cancer, and 9 for lung cancer markers (Table 1).

Table 1.

Cancer Type	Accession	Name	Description	Seq, ID No.
PR	NM_001648	KLK3	kallikrein 3, (prostate specific antigen)	Seq. ID No. 1
PR	NM_005551	KLK2	kallikrein 2, prostatic	Seq. ID No. 2
BR	NM_004064	CDKN1B	cyclin-dependent kinase inhibitor 1B (p27, Kip1)	Seq. ID No. 34
BR	NM_002411	MGB1	mammaglobin 1	Seq. ID No. 3
BR	NM_005264	GFRA1	GDNF family receptor alpha 1	Seq. ID No. 4
BR	None	C18ORF1	chromosome 18 open reading frame 1	Seq. ID No. 98
BR	NM_000095	COMP	cartilage oligomeric matrix protein	Seq. ID No. 67
CO	NM_001804	CDX1	caudal type homeo box transcription factor 1	Seq. ID No. 8
CO	NM_001046	SLC12A2	solute carrier family 12 (sodium/potassium/ chloride transporters), member 2	Seq. ID No 9
CO	NM_001285	CLCA1	chloride channel, calcium activated, family member 1	Seq. ID No. 11
CO	NM_007052	NOX1	NADPH oxidase 1	Seq. ID No. 13
CO	NM_002457	MUC2	mucin 2, intestinal/tracheal	Seq. ID No. 14
CO	NM_004063	CDH17	cadherin 17, LI cadherin	Seq. ID No 15
LU_A	NM_021950	MS4A2	membrane-spanning 4-domains, subfamily A, member 2	Seq. ID No. 17

Table 1. (continued)

Cancer Type	Accession	Name	Description	Seq, ID No.
LU_A	NM_000964	ASAHL	N-acylsphingosine amidohydrolase (acid ceramidase)-like	Seq. ID No. 18
LU_A	NM_006495	EVI2B	ecotropic viral integration site 2B	Seq. ID No. 20
LU_A	NM_006864	LILRB3	leukocyte immunoglobulin-like receptor, subfamily B	Seq. ID No. 21
LU_A	X67301	none	H.sapiens mRNA for IgM heavy chain constant region (Ab63)	Seq. ID No. 22
LU_A	NM_002123	HLA-DQB1	major histocompatibility complex, class II, DQ beta 1	Seq. ID No.23
LU_S	NM_000673	ADH7	alcohol dehydrogenase 7 (class IV), mu or sigma polypeptide	Seq. ID No. 24
LU_S	NM_003722	TP63	tumor protein 63 kDa with strong homology to p53	Seq. ID No. 26
LU_S	None	SOX2	SRY (sex determining region Y)-box 2	Seq. ID No. 32
OV	NM_000906	NPR1	natriuretic peptide receptor A/guanylate cyclase A	Seq. ID No. 28
OV	NM_000378	WT1	Wilms tumor 1	Seq. ID No. 30

Example 2: Heuristic Step

[0045] A heuristic rule was further applied to the portfolio obtained in Example 1. That is, the rule stated that if the gene/marker identified would likely be expressed in peripheral blood or were well-characterized tissue markers (e.g. PSA, mammaglobin, etc.), then such genes/marker would be removed from the portfolio. Application of the rule enabled the establishment of a portfolio of genes/markers that are optimized for use in a screening application in which the patient sample is obtained by assaying components found in the peripheral blood such as epithelial cells. The result of the selected portfolio contains 31 genes as shown in Table 2.

Table 2.

Cancer Type	Accession	Name	Description	Seq. ID No.
PR	Hs.12784	KIAA0293	KIAA0293 protein	Seq. ID No. 67
PR	NM_006562	LBX1	transcription factor similar to D. melanogaster homeodomain protein lady bird late	Seq. ID No. 33
PR	NM_016026	LOC51109	CGI-82 protein	Seq. ID No. 34
PR	HG2261-HT2352	none	Antigen	Seq. ID No. 99
PR	NM_012449	STEAP	six transmembrane epithelial antigen of the prostate	Seq. ID No. 35
PR	NM_001634	AMD1	S-adenosylmethionine decarboxylase 1	Seq. ID No. 36
PR	HG2261-HT2351	none	Antigen I	Seq. ID No. 100
PR	NM_006457	LIM	LIM protein (similar to rat protein kinase C-binding enigma)	Seq. ID No. 37
BR	NM_005853	IRX5	iroquois homeobox protein 5	Seq. ID No. 38
BR	NM_005264	GFRA1	GDNF family receptor alpha 1	Seq. ID No. 39
BR	none	C180RF1	chromosome 18 open reading frame 1	Seq. ID No. 98

Table 2. (continued)

Cancer Type	Accession	Name	Description	Seq. ID No.
BR	NM_000095	COMP	cartilage oligomeric matrix protein (pseudoachondroplasia, epiphyseal dysplasia 1, multiple)	Seq. ID No. 41
CO	NM_001265	CDX2	caudal type homeo box transcription factor 2	Seq ID No. 43
CO	NM_001046	SLC12A2	solute carrier family 12 (sodium/potassium/ chloride transporters), member 2	Seq. ID No. 44
CO	NM_001285	CLCA1	chloride channel, calcium activated, family member 1	Seq. ID No. 46
CO	NM_004063	CDH17	cadherin 17, LI cadherin (liver-intestine)	Seq. ID No.48
OV	NM_000906	NPR1	natriuretic peptide receptor A/guanylate cyclase A (atrionatriuretic peptide receptor A)	Seq. ID No. 50
OV	NM_005504	BCAT1	branched chain aminotransferase 1, cytosolic	Seq. ID No. 52
OV	NM_002398	MEIS1	Meis1 (mouse) homolog	Seq. ID No. 53
OV	none	SPON1	spondin 1, (f-spondin) extracellular matrix protein	Seq. ID No. 69
OV	NM_001692	none	M25809:Human endomembrane proton pump subunit mRNA IGenBank=M25809	Seq. ID No. 54
OV	NM_002774	KLK6	kallikrein 6 (neurosin, zyme)	Seq. ID No. 55
LU_A	NM_000964	ASAHL	N-acylsphingosine amidohydrolase (acid ceramidase)-like	Seq. ID No. 56
LU_A	NM_002838	PTPRC	protein tyrosine phosphatase, receptor type, C	Seq. ID No. 58
LU_A	NM_015364	MD-2	MD-2 protein	Seq. ID No. 59
LU_A	NM_006875	PIM2	pim-2 oncogene	Seq. ID No. 60
LU_S	NM_005554	KRT6A	keratin 6A	Seq. ID No. 61
LU_S	NM_000673	ADH7	alcohol dehydrogenase 7 (class IV), mu or sigma polypeptide	Seq. ID No. 62
LU_S	NM_003722	TP63	tumor protein 63 kDa with strong homology to p53	Seq. ID No. 64
LU_S	none	SOX2	SRY (sex determining region Y)-box 2	Seq. ID No. 32
LU_S	NM_005688	ABCC5	ATP-binding cassette, sub-family C (CFTR/ MRP), member 5	Seq. ID No. 66

Example 3: Prognostic Portfolios

[0046] A patient sample set with known clinical outcomes was used to test the portfolio selection method of the invention. The sample set is described in van't Veer, L. J et al. Gene Expression Profiling Predicts Clinical Outcome of Breast Cancer, *Nature*, 415, 530 - 536, (2002), incorporated herein by reference. In that study, breast tissue samples were obtained from 78 patients exhibiting sporadic breast tumors. The patients were all less than 55 years of age and presented with a tumor less than 5cm. All were lymph node negative. Thirty four of the patients presented with distant metastases in less than 5 years while 44 showed no distant metastases in the same period.

[0047] The data from the study were then processed according to the method of the invention. Sample number 54 was removed from further analysis due to a high percentage of missing values. The mean and standard deviation of

the intensity measurements for each gene were calculated using the non-metastatic samples as the baseline. A discriminating value of X*(Standard Deviation + Mean) was then calculated for each baseline gene (X was assigned a value of 3). This value was used to ensure the resulting portfolio would be stringent. A ratio of the discriminating value to the baseline value was then calculated for each metastatic sample. This ratio was then converted to a common logarithm. This data was then imported into Wagner Software which produced an efficient frontier from which a portfolio of 16 genes was selected. The baseline and experimental values were then reversed and a second portfolio of 12 markers representing genes up-regulated in the non-metastatic cases was produced. The second portfolio's value is subtracted from the first portfolios value to create a combined portfolio value from all 28 genes. This final portfolio is comprised of genes from Seq. ID No.70 -97. 17 of the genes of this portfolio were also present in the 70 gene portfolio described in the reference. The genes of the portfolio are identified below. (Seq. ID No. 70, Seq. ID No. 72, Seq. ID No. 73-77, Seq. ID No. 79, Seq. ID No. 80, Seq. ID No. 85, Seq. ID No. 87, Seq. ID No. 91-93, Seq. ID No. 95 and Seq. ID. No. 97.)

28 Gene list (2 Portfolios) Up in Metastatic Patients (Portfolio 1)	
Contig53226_RC	Seq. ID No. 89
NM_012214	Seq. ID No. 82
NM_020386	Seq. ID No. 86
NM_004504	Seq. ID No. 81
AA555029_RC	Seq. ID No. 70
AL080059	Seq. ID No.74
AF055033	Seq. ID No. 73
NM_016448	Seq. ID No. 85
Contig40831_RC	Seq. ID No. 95
Contig63649_RC	Seq. ID No. 91
Contig24252_RC	Seq. ID No. 93
NM_000436	Seq. ID No. 75
NM_002019	Seq. ID No. 77
Contig55313_RC	Seq. ID No. 90
Contig25991	Seq. ID No. 97
NM_000788	Seq. ID No. 76
Up in Non-Metastatic Patients (Portfolio 2) AB033007	Seq. ID No. 71
Contig42421_RC	Seq. ID No. 96
NM_003748	Seq. ID No. 78
NM_013262	Seq. ID No. 83
NM_003862	Seq. ID No. 79
NM_003882	Seq. ID No. 80
Contig48328_RC	Seq. ID No. 87
NM_015416	Seq. ID No. 84
AB037863	Seq. ID No. 72
Contig27312_RC	Seq. ID No. 88
Contig32125_RC	Seq. ID No. 92
Contig49670_RC	Seq. ID No. 94

17 Overlap	
Systematic name	
NM_003862	Seq. ID No. 79
NM_003882	Seq. ID No. 80
Contig48328_RC	Seq. ID No. 87
AA555029_RC	Seq. ID No. 70
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AF055033	Seq. ID No.73
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Contig32125_RC	Seq. ID No. 92
Contig25991	Seq. ID No. 97
NM_000788	Seq. ID No. 76

[0048] The two portfolios were then used to determine the prognosis of the 78 original samples by comparing gene expression signatures from the microarray data according to the method for testing the classification accuracy described in the reference. In the case of the 70 gene portfolio, 81% of the samples were properly characterized according to an optimized threshold biased to include ambiguous signatures as indicative of poor prognosis (85% for an absolute threshold). This portfolio misclassified 3 patients with a poor prognosis as having a good prognosis using the optimized threshold (5 for the absolute threshold). Twelve patients with a good prognosis were misclassified as having a good prognosis when they had a bad prognosis using the optimized threshold (8 for absolute).

[0049] In the case of the 28 gene portfolio, 94% of the samples were properly characterized according to an optimized threshold biased to include ambiguous signatures as indicative of poor prognosis (93% for an absolute threshold). This portfolio misclassified 3 patients with a poor prognosis as having a good prognosis using the optimized threshold (5 for the absolute threshold). Three patients with a good prognosis were misclassified as having a good prognosis when they had a bad prognosis using the optimized threshold (2 for absolute).

[0050] Comparing the two profiles, it is apparent that the profiles selected according to the method of the invention are much more economical and produce results that are more accurate and reliable than those of the comparative portfolio.

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Claims

1. A method of selecting a portfolio of markers for use in a diagnostic application comprising:

   a) defining diagnostic parameters,
   b) establishing a relationship among the diagnostic parameters that identifies an optimized portfolio of markers, and
   c) selecting said portfolio.

2. The method of claim 1 wherein the markers comprise genes.

3. The method of claim 2 wherein the relationship is based on the expression of the gene.

4. The method of claim 2 wherein the relationship is based on the variance of the expression of the gene.

5. The method of claim 4 wherein the diagnostic parameters include a measure of the relative degree of expression of a gene and a measure of the variation in the measurement of the degree of expression of the gene; and the relationship is a mean variance relationship.

6. The method of claim 1 further comprising the application of a heuristic rule.

7. The method of claim 1 wherein the diagnostic parameters represent sensitivity and specificity.

8. A machine comprising a general purpose computer programmed to identify a portfolio of markers wherein said machine comprises instructions to employ a relationship among diagnostic parameters so that markers are optimized.

9. The machine of claim 8 programmed to operate according to the method of any one of claims 1 to 7

10. An article comprising instructions for selecting a portfolio of markers for use in a diagnostic application comprising:

    a) instructions for inputting diagnostic parameters into an algorithm,
    b) instructions for operating said algorithm wherein said algorithm relates the diagnostic parameters such that an optimized portfolio of markers is identifiable, and
    c) instructions for selecting said portfolio.

11. The article of claim 10 which are adapted for performance of the method of any one of claims 1 to 7

12. The article of claim 10 or claim 11 wherein the instructions are machine readable.

13. The article of claim 12 wherein the instructions are computer readable.

14. A diagnostic portfolio selected according to the method of any one of claims 1 to 7.

15. The portfolio of claim 14 comprising genes.

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Office européen des brevets

(11) EP 1 349 104 A3

(12) EUROPEAN PATENT APPLICATION

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01.10.2003 Bulletin 2003/40

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(71) Applicant: **Ortho-Clinical Diagnostics, Inc.**
Rochester, NY 14626-5101 (US)

(72) Inventor: **Jatkoe, Tim**
La Jolla, CA 92037-4335 (US)

(74) Representative: **Mercer, Christopher Paul et al**
Carpmaels & Ransford
43, Bloomsbury Square
London WC1A 2RA (GB)

(54) Method of selecting a portfolio of markers for use in a diagnostic application

(57) Methods of selecting a portfolio of markers for use in a diagnostic applications include defining diagnostic parameters, establishing a relationship among the parameters so that they are optimized, and selecting an optimal group of markers for the diagnostic application. The diagnostic parameters can include a measure of the relative degree of expression of a gene, a measure of the variation in the measurement of the degree of expression of the gene, and the relationship between the diagnostic and discriminating parameters can be a mean variance relationship.

Machines programmed to conduct the method and articles that comprise instructions for their operation are further aspects of the invention.

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European Patent Office

PARTIAL EUROPEAN SEARCH REPORT

which under Rule 45 of the European Patent Convention shall be considered, for the purposes of subsequent proceedings, as the European search report

Application Number EP 03 25 2027

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.7)
X,D	VAN'T VEER LAURA J ET AL: "Gene expression profiling predicts clinical outcome of breast cancer" NATURE (LONDON), vol. 415, no. 6871, 31 January 2002 (2002-01-31), pages 530-536, XP002259781 ISSN: 0028-0836 * the whole document * ---	1-4,6-13	G06F19/00
X,D	SU A I ET AL: "Molecular classification of human carcinomas by use of gene expression signatures" CANCER RESEARCH, AMERICAN ASSOCIATION FOR CANCER RESEARCH, BALTIMORE, MD, US, vol. 61, 15 October 2001 (2001-10-15), pages 7388-7393, XP002242441 ISSN: 0008-5472 * the whole document * --- -/--	1-4,6-13	

TECHNICAL FIELDS SEARCHED (Int.Cl.7)

G06F

INCOMPLETE SEARCH

The Search Division considers that the present application, or one or more of its claims, does/do not comply with the EPC to such an extent that a meaningful search into the state of the art cannot be carried out, or can only be carried out partially, for these claims.

Claims searched completely :

Claims searched incompletely :

Claims not searched :

Reason for the limitation of the search:

see sheet C

Place of search	Date of completion of the search	Examiner
MUNICH	31 October 2003	Luo, X

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

EPO FORM 1503 03.82 (P04C07)

European Patent Office

INCOMPLETE SEARCH
SHEET C

Application Number
EP 03 25 2027

Claim(s) searched incompletely:
1-15

Reason for the limitation of the search:

The initial phase of the search revealed a very large number of documents relevant to the issue of novelty. So many documents were retrieved that it is impossible to determine which parts of the claim(s) may be said to define subject-matter for which protection might legitimately be sought (Article 84 EPC). For these reasons, a meaningful search over the whole breadth of the claim(s) is impossible. Consequently, the search has been restricted to the examples 1-3.

Present claims 14-15 relate to a portfolio defined by reference to a desirable characteristic or property, namely being selected according to the method of any one of claims 1-7.

The claims cover all portfolios having this characteristic or property, whereas the application provides support within the meaning of Article 84 EPC and/or disclosure within the meaning of Article 83 EPC for only a very limited number of such portfolios. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Independent of the above reasoning, the claims also lack clarity (Article 84 EPC). An attempt is made to define the portfolios by reference to a result to be achieved. Again, this lack of clarity in the present case is such as to render a meaningful search over the whole of the claimed scope impossible. Consequently, the search has been carried out for those parts of the claims which appear to be clear, supported and disclosed, namely the portfolios disclosed in the examples.

European Patent Office

PARTIAL EUROPEAN SEARCH REPORT

Application Number
EP 03 25 2027

DOCUMENTS CONSIDERED TO BE RELEVANT			CLASSIFICATION OF THE APPLICATION (Int.Cl.7)
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	
X	GRUVBERGER S ET AL: "Estrogen receptor status in breast cancer is associated with remarkable distinct gene expression patterns" CANCER RESEARCH, AMERICAN ASSOCIATION FOR CANCER RESEARCH, BALTIMORE, MD, US, vol. 61, no. 16, 15 August 2001 (2001-08-15), pages 5979-5984, XP002964618 ISSN: 0008-5472 * the whole document * ---	1-4,6-13	
X	KHAN JAVED ET AL: "Classification and diagnostic prediction of cancers using gene expression profiling and artificial neural networks" NATURE MEDICINE, vol. 7, no. 6, June 2001 (2001-06), pages 673-679, XP001155989 ISSN: 1078-8956 * abstract * ---	1-4,6-13	TECHNICAL FIELDS SEARCHED (Int.Cl.7)
A	DANIEL H. WAGNER, ASSOCIATES, INC.: "M-V Optimizer USER'S GUIDE" INTERNET, [Online] 1998, XP002259783 Retrieved from the Internet: <URL:http://web.archive.org/web/20011202011232/www.mathfinance.wagner.com/PRODUCTS/MVOPT/WAoptimize/waoptimize.html> [retrieved on 2003-10-29] * the whole document * ---		
	-/--		

EPO FORM 1503 03.82 (P04C10)

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PARTIAL EUROPEAN SEARCH REPORT

Application Number
EP 03 25 2027

DOCUMENTS CONSIDERED TO BE RELEVANT			CLASSIFICATION OF THE APPLICATION (Int.Cl.7)
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	
A	RUSTEM B ET AL: "ROBUST MINI-MAX PORTFOLIO STRATEGIES FOR RIVAL FORECAST AND RISK SCENARIOS" JOURNAL OF ECONOMIC DYNAMICS AND CONTROL, AMSTERDAM, NL, vol. 24, no. 11/12, October 2000 (2000-10), pages 1591-1621, XP001155000 * the whole document * ---		TECHNICAL FIELDS SEARCHED (Int.Cl.7)

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